

NF- κ B Activation in Microglia Induces Motor Neuron Death

in Amyotrophic lateral sclerosis

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ALS is a fatal neurodegenerative disease that affects motor neurons (MNs) resulting in severe muscle atrophy and paralysis. Despite the fact that about 5,600 people are diagnosed with ALS in the US each year, there is no cure. One of the most striking pathological hallmarks observed in patients and rodent models of ALS is neuroinflammation, characterized by activation of microglia, the resident immune cells of the central nervous system. Genetic approaches to globally eliminate single inflammatory genes in ALS mouse models have largely failed, highlighting the complexity of this inflammatory process. Nuclear Factor-kappa B (NF- κ B) regulates many pro-inflammatory mediators found in areas of MN death in ALS, and this pathway is activated in the spinal cords of ALS patients. Therefore, the purpose of this study is to determine the role of NF- κ B, a master regulator of inflammation, in ALS. By immunoblot and immunohistochemical analyses, we determined NF- κ B activation increases with disease progression in ALS mice and occurs predominantly in microglia. To determine the role of NF- κ B activation in microglial-mediated MN death, we utilized an *in vitro* co-culture model in which 61% of MNs die when co-cultured with ALS microglia after 72 hours. NF- κ B inhibition in ALS microglia fully rescued MNs from microglial-mediated death *in vitro*. Remarkably, conditional inhibition of NF- κ B in microglia *in vivo* extends survival in the ALS mice by delaying disease progression by 51%, one of the longest extensions reported in this severe model. NF- κ B inhibition in microglia leads to marked reduction in prototypic inflammatory markers such as CD68, CD86 and iNOS, suggesting that NF- κ B regulates microglial conversion to a pro-inflammatory, neurotoxic state in ALS. Furthermore, we show that constitutive activation of NF- κ B selectively in microglia in wild-type mice promoted a similar microglial activation state observed in ALS mice. Strikingly, microglia isolated from these constitutively active NF- κ B mice rapidly induced 48% MN death *in vitro*. These data provide a

cellular and molecular mechanism by which microglia induce MN death in ALS and suggest a novel therapeutic target to modulate microglial activation and slow the progression of ALS and other neurodegenerative diseases by which microglial activation plays a role.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a devastating, fast-progressing neurodegenerative disease that causes death of motor neurons in the brainstem, spinal cord, and motor cortex. Typically within 2-5 years of clinical onset, patients succumb to the disease due to severe muscle atrophy, paralysis, and ultimately denervation of respiratory muscles. Most ALS cases are classified as sporadic, defined as having no familial history of the disease. The remaining 5-10% of cases are classified as familial and are typically inherited in an autosomal dominant fashion. Despite genetic differences, familial and sporadic ALS are clinically indistinguishable. About 20% of familial cases are associated with mutations in the gene superoxide dismutase 1 (SOD1) (Rosen et al., 1993). Transgenic rodents carrying mutant forms of SOD1 develop a similar, progressive motor neuron disease as patients and are used as the gold standard in ALS research. Elegant work utilizing these animals has revealed that non-neuronal cells play a crucial role in ALS contributing to motor neuron death via a non-cell autonomous mechanism. In chimeric mice expressing mixtures of mutant SOD1-expressing cells and wild-type cells, wild-type motor neurons exhibited signs of ALS pathology when surrounded by mutant SOD1 glia (Clement, 2003). Similarly, *in vitro* studies have shown that murine astrocytes and microglia expressing mutant SOD1 as well as human astrocytes from sporadic and familial ALS patients can induce motor neuron death (Giorgio et al., 2007; Haidet-Phillips et al., 2011; Nagai et al., 2007; Xiao et al., 2007). Evidence that multiple cell types mediate different aspects of disease emerged from generation of mice expressing a floxed mutant SOD1 gene. Removal of the floxed mutant SOD1 gene specifically in motor neurons extended survival by delaying disease onset and early disease progression, while excising floxed mutant SOD1 in either microglia/macrophages or astrocytes extended survival by slowing disease progression, but not onset (Beers et al., 2006; Boillee et al., 2006; Yamanaka et al., 2008). Similarly, when the myeloid lineage of

mutant SOD1 mice was replaced with wild-type microglia and macrophages, disease onset was not altered, but disease progression was slowed (Beers et al., 2006). This suggests ALS is a deadly convergence of damage developed in multiple cellular compartments that ultimately leads to neuromuscular failure. However, the precise mechanisms by which the individual cell types such as astrocytes and microglia contribute to the disease remain unknown.

Extensive astrocyte and microglial activation and infiltration of peripheral immune cells at sites of motor neuron degeneration is one of the most striking hallmarks of ALS shared by familial and sporadic patients as well as rodent models (Hall et al., 1998; Kawamata et al., 1992; Mantovani et al., 2009). Mutant SOD1 astrocytes and microglia exhibit increased expression of many pro-inflammatory genes including inducible nitric oxide synthase (iNOS), NADPH oxidase (NOX2), TNF- α , prostaglandin E₂, COX-2 and IL-1 β (Henkel et al., 2006; Hensley et al., 2006; 2003; 2002; Sasaki et al., 2001; Weydt and Möller, 2005). However, genetic approaches to globally eliminate single inflammatory genes in ALS mouse models have largely failed to extend survival (Almer et al., 2006; Gowing et al., 2006; Nguyen et al., 2001; Son et al., 2001) and in some cases have even hastened disease progression (ref gal3 paper). This suggests inflammation in ALS is a combination of compensatory mechanisms acting in a redundant manner to induce motor neuron death. Additionally, inflammation may play opposing roles in different cell types and this may change throughout the course of disease. For these reasons it is necessary to study the intricate molecular mechanisms driving the inflammatory response in each cellular compartment and to identify up-stream transcription factors that are dysregulated with disease in individual cell types.

Nuclear Factor- κ B (NF- κ B) is known to be a major regulator of inflammation, regulating gene expression of pro-inflammatory cytokines, chemokines, enzymes and adhesion molecules, many of which are upregulated in ALS. When inflammatory mediators bind their respective receptors, it initiates a signaling cascade that leads to phosphorylation and activation of IKK β (a subunit of the inhibitor of κ B kinase, IKK, complex). Activated IKK β phosphorylates the I κ B inhibitory proteins, targeting them for ubiquitination and proteosomal degradation, and releases the NF- κ B complex (p65-p50) to be

phosphorylated and translocated to the nucleus to induce gene expression (Ghosh and Karin, 2002). We previously reported NF- κ B as the highest-ranked regulator of inflammation by Ingenuity Pathway analysis of inflammatory gene array data from astrocytes derived from human post mortem ALS patients (Haidet-Phillips et al., 2011). Other laboratories have confirmed by immunohistochemistry that NF- κ B is activated in glia in familial and sporadic ALS patients (Migheli et al., 1997; Swarup et al., 2011). Interestingly, loss of function mutations in the gene optineurin, which is known to negatively regulate TNF- α -induced NF- κ B activation, have been found in ALS patients (Maruyama et al., 2010). However, it remains unknown whether NF- κ B activation in ALS glia is involved in motor neuron death.

Since the role of glial-derived inflammation in ALS remains ambiguous, we sought to determine how pro-inflammatory signaling regulated by NF- κ B in astrocytes and microglia mediates motor neuron death in ALS. In the current study we demonstrate that NF- κ B is activated with disease progression in the SOD1-G93A mouse model predominately in glia. Surprisingly, inhibition of NF- κ B in astrocytes using transgenic and viral-mediated gene delivery approaches did not increase motor function or survival in SOD1-G93A mice. However, crossing the SOD1-G93A mouse model to a NF- κ B-GFP reporter strain revealed NF- κ B activation predominately occurs in microglia. We developed a robust *in vitro* co-culture model of ALS utilizing adult primary microglia and embryonic stem cell-derived motor neurons. Using this *in vitro* system we show that mutant SOD1 microglia reduce MN survival by 61%. Notably, inhibition of NF- κ B in ALS microglia fully rescued motor neurons in the *in vitro* co-culture model. Consistently with these results, inhibition of NF- κ B specifically in microglia and macrophages *in vivo* significantly delayed disease progression in the SOD1-G93A mouse model by 51%. This is one of the largest extensions in survival observed in this severe mouse model. Inhibition of Nf κ B in microglia leads to marked reduction in prototypic inflammatory markers such as CD68, CD86 and iNOS, suggesting that NF- κ B inhibition blocks microglial conversion to a pro-inflammatory, neurotoxic state. Furthermore, we show that constitutive activation of NF- κ B selectively in microglia in wild-type mice promoted a similar microglial activation state observed in the SOD1-G93A mice. Strikingly, microglia isolated from these

animals rapidly induced motor neuron death *in vitro*. Taken together, these data provide a cellular and molecular mechanism by which microglia induce motor neuron death in ALS and suggest a new therapeutic target to modulate microglial activation and slow the progression of ALS and other neurodegenerative diseases by which microglial activation plays a role.

RESULTS

NF-κB is activated with disease progression in the SOD1-G93A mouse model of ALS

To investigate the extent of NF-κB activation in the SOD1-G93A model at different stages of disease, we analyzed lumbar spinal cord protein for phospho-p65 (active form of NF-κB) from four SOD1-G93A mice at 60, 80, 100, and 120 days of age as well as end-stage (typically around 130 days in males and 140

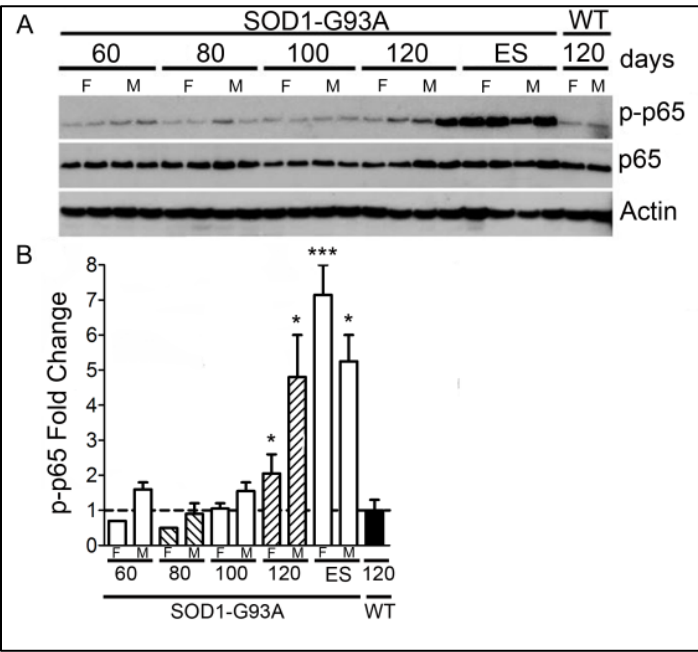


Figure 1. NF-κB is activated with disease progression in the SOD1-G93A mouse model of ALS.

- (A) Immunoblot of lumbar spinal cord protein isolated from wild-type mice at 120 days of age (n=2) and from SOD1-G93A mice at 60, 80, 100, 120 days of age and at end-stage (ES) shows increase in phosphorylation of p65 with disease progression (top). The blot was reprobed for p65 (middle) and Actin (bottom) as loading controls. n=4 for each time point.
- (B) Fold change of the immunoblot in (A). Phospho-p65 was found to be significantly upregulated by 2 and 4.8 fold in females and males respectively at 120 days and by 7 and 5 fold at end stage in females and males respectively. Band intensities were normalized to p65/Actin.

days in females). As disease progresses phospho-p65 levels increased two-fold at 120 days of age in females and 4.8 fold in males. At end stage of disease phospho-p65 levels increased 7 fold in females and 5 fold in males.

NF-κB activation occurs predominately in microglia in the SOD1-G93A mouse model

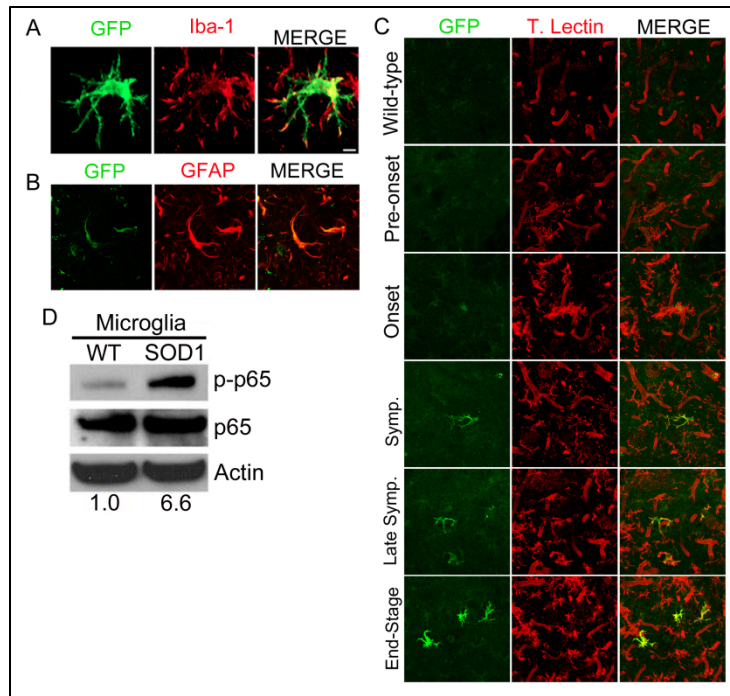


Figure 2. NF-κB activation occurs predominately in microglia in the SOD1-G93A mouse model

(A and B) representative high magnification images of the cell type positive for NF-κB-GFP (green) in the lumbar ventral horn of a 120 day old SOD1-G93A; NF-κB-GFP mouse. Most predominant GFP+ cells (A) also positive for microglial marker Iba1+ (red). Other less-striking GFP+ cells positive for astrocyte marker GFAP (blue).

(C) Representative lumbar ventral horn images of WT; NF-κB-GFP at 120 days, and SOD1-G93A; NF-κB-GFP mice at 60, 80, 100, 120, and 130 days (end-stage) show increase in NF-κB activation (GFP) throughout disease progression in SOD1-G93A microglia labeled by tomato lectin (red).

(D) Immunoblot of protein isolated from primary microglia obtained from 120 day old SOD1-G93A mice and WT littermates confirm NF-κB is activated 6.6 fold in SOD1-G93A microglia compared to control littermates. Microglia from 6 mice were pooled together for protein isolation. Fold change determined by phospho-p65 band intensity normalized to p65/Actin.

To, evaluate the cell types contributing to the increase in lumbar NF-κB activation, we crossed the SOD1-G93A mice to a an NF-κB-GFP reporter mouse strain that expresses GFP under the control of NF-κB *cis* elements (Magness et al., 2004). Since robust NF-κB activation in SOD1-G93A was evident at 120 days in lumbar spinal cord protein, we analyzed lumbar spinal cord sections from 120 day old SOD1; NF-κB-GFP mice for GFP expression. We observed a population of bright GFP+ cells identified as microglia by overlapping Iba-1 staining (Figure 2A). We also observed a dim GFP+ population of GFAP+ astrocytes (Figure 2B). We confirmed these findings by analyzing phospho-p65 levels in protein from microglia isolated from 120 day old SOD1-G93A mice. Phospho-p65 was 6.6 fold greater in SOD1-G93A microglia than WT microglia (Figure 2D). To determine the time course of NF-κB activation in microglia as disease progresses, we performed immunohistochemistry of SOD1; NF-κB-GFP lumbar spinal cord sections prior to disease onset (60 days), at disease onset (100 days), during disease progression (120 days), and at end-stage (130 days). We observed faint GFP signal that co-localized with microglial marker, tomato lectin, at 60 days. There was a marked increase in GFP+ cells beginning at

disease onset progressing up to end-stage that co-stained with tomato lectin suggesting NF- κ B activation coincides with microgliosis (Figure 2C). This data revealed that microglia, not astrocytes, are the prominent cell type contributing to NF- κ B activation in disease progression.

Adult SOD1-G93A microglia induce motor neuron death in an NF- κ B dependent mechanism *in vitro*.

To determine whether NF- κ B activation in microglia is involved in motor neuron death in an *in vitro* co-culture model of ALS, we employed two independent approaches to abolish NF- κ B activation in microglia. First, we overexpressed DN-ikB α via adenovirus in SOD1-G93A and wild-type microglia. We also employed a genetic approach by isolating microglia from SOD1-G93A; IKK $\beta^{fl/fl}$ mice and infecting the microglia *in vitro* with an adenovirus expressing cre recombinase to remove IKK $\beta^{fl/fl}$ in microglia post-

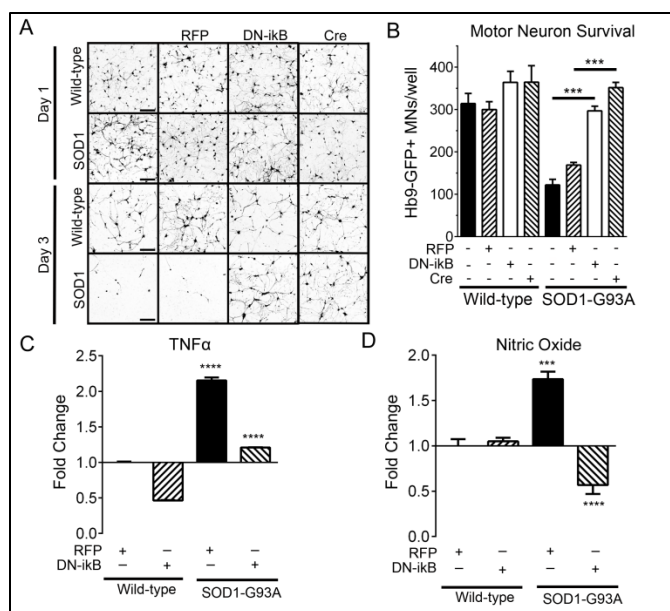


Figure 3. SOD1-G93A microglia induce MN death in an NF- κ B-dependent mechanism

(A and B) Representative microscopic fields (A) and entire well counts (B) of Hb9-GFP+ motor neurons after 12 hours and 72 hours in co-culture with WT or SOD1-G93A microglia not infected (black bars) or infected with Ad-RFP (dashed bars) or Ad-DNikB α (white bars) show NF- κ B inhibition rescues motor neuron survival. Co-culturing MNs with either WT;IKK $\beta^{fl/fl}$ or SOD1-G93A; IKK $\beta^{fl/fl}$ microglia infected with Ad-cre (dashed bars) confirmed this result. (C and D) Quantification of nitric oxide (C) and TNF- α (D) in the co-culture medium by ELISA confirms SOD1-G93A inflammation was dampened with NF- κ B inhibition.

isolation. After 12 hours of co-culture with SOD1-G93A microglia, we observed no difference in motor neuron survival or axon length of the motor neurons compared to WT controls (Figure 3A). However, after 72 hours of co-culture we observed a 61% reduction in motor neuron survival and marked reduction in axon length when motor neurons were co-cultured with SOD1-G93A microglia compared to WT (Figure 3B). Inhibiting NF- κ B, either transgenically or by overexpression of DN-ikB α , fully rescued motor neuron axon length and survival *in vitro* to wild-type levels (Figure

3A and 3B). We evaluated the efficiency of NF- κ B inhibition by measuring nitric oxide (NO) and TNF- α levels in the co-culture medium. When NF- κ B was inhibited, nitric oxide levels decreased 1.75 fold to 0.5 fold compared to WT (Figure 3C). TNF- α levels were reduced 2 fold compared to WT (Figure 3D). These data suggest that SOD1-G93A microglia induce motor neuron death in an NF- κ B dependent mechanism.

Conversely, to evaluate whether constitutively activating NF- κ B in WT microglia is sufficient to induce motor neuron death *in vitro*, we isolated microglia from mice containing inducible constitutively active IKK β (IKK β CA) when cre is expressed.

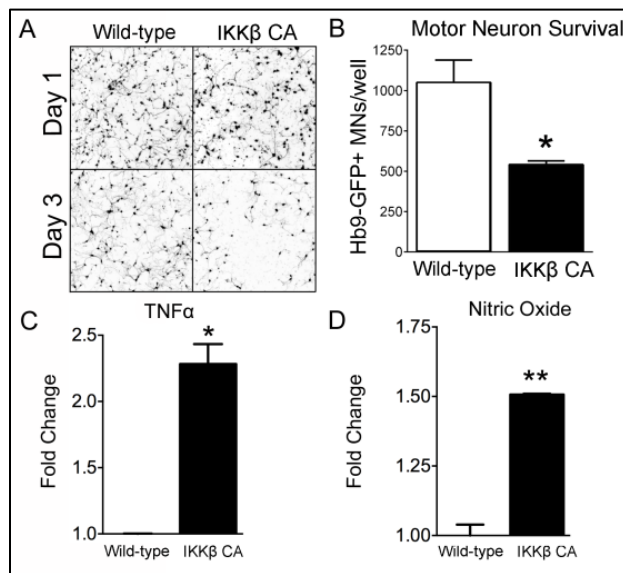


Figure 4. Wild type microglia induce motor neuron death when NF- κ B is constitutively active. (A and B) Representative microscopic fields (A) and entire well counts (B) of Hb9-GFP+ MNs after 12 and 72 hours in co-culture with WT; R26Stop^{fl} IKK β CA microglia uninfected or infected with Ad-cre to induce expression of constitutively active IKK β shows constitutive activation of NF- κ B in normal microglia induces motor neuron death. (C and D) Quantification of nitric oxide (C) and TNF- α (D) in the co-culture medium by ELISA

Post-isolation we infected microglia from these mice with Ad-cre to induce transcription of IKK β CA. Uninfected microglia from the same isolation were used as WT controls. After 12 hours in co-culture with WT or IKK β CA microglia, we observed no difference in motor neuron axon length or survival (Figure 4A). Interestingly, after 72 hours in co-culture IKK β CA microglia induced a 50% statistical decrease in motor neuron survival compared to controls (Figure 4B). These data suggests that NF- κ B activation in microglia is sufficient to induce motor neuron death independent of the SOD1-G93A mutation.

SOD1-G93A microglia induce motor neuron death in an NF- κ B dependent mechanism *in vivo*.

Since we have established that (1) NF- κ B activation during the disease course in SOD1-G93A mice occurs predominantly in microglia (Figure 2) and (2) SOD1-G93A microglia utilize an NF- κ B-dependent mechanism to induce motor neuron death *in vitro* (Figure 3), we sought to determine how NF- κ B inhibition in microglia affects the disease course in the SOD1-G93A mouse model. To accomplish this we crossed SOD1-G93A; IKK $\beta^{f/f}$ mice to mice expressing cre recombinase driven by the promoter for the gene *c-fms* which encodes Colony stimulating factor receptor 1 (CSF-1R). As shown by MacGreen reporter mice that express GFP under the regulation of the *c-fms* promoter, only microglia express CSF-1R in the postnatal mouse brain (Erblich et al., 2011). To confirm cell-type specificity of cre expression driven by the *c-fms* (CSF-1R) promoter, we crossed CSF-1R-cre mice to the Rosa26-TdTomato mouse strain that expresses RFP in all cre-expressing cells. We observed RFP expression only in Iba-1+ microglia in the adult mouse spinal cord, and RFP expression was absent in neurons or astrocytes (data not shown).

Wild-type and SOD1 CSF-1R-cre+ mice homozygous for IKK $\beta^{f/f}$ displayed serious immune dysfunction such as enlarged spleens, eye infections, and missing or very brittle teeth, and therefore could not be maintained in the colony long enough to evaluate survival. Thus, we analyzed mice heterozygous for the floxed IKK β allele (IKK $\beta^{f/wt}$). To determine the efficiency of IKK β knockdown in heterozygous mice, we performed immunohistochemistry for IKK β in lumbar spinal cord sections from SOD1-G93A; IKK $\beta^{f/wt}$; CSF-1R-cre+ and cre- mice. SOD1-G93A; IKK $\beta^{f/wt}$; CSF-1R-cre+ showed a decrease in IKK β staining compared to cre negative controls (data not shown). To ensure knockdown was specific for IKK β , we also evaluated IKK γ levels and observed no difference between cre+ and cre- mice. To confirm that decreasing IKK β levels would lead to a decrease in NF- κ B activation, we analyzed lumbar spinal cord protein for phospho-p65 from SOD1-G93A; IKK $\beta^{f/wt}$; CSF-1R-cre+ or cre- mice at end-stage. Phospho-p65 levels in SOD1-G93A; cre- controls were 8.2 fold greater than WT controls. Importantly, phospho-p65 was reduced 45% in SOD1-G93A; IKK $\beta^{f/wt}$; CSF-1R-cre+ mice compared to cre- controls. This reduction in NF- κ B activation resulted in a 21 day extension in median survival in SOD1-G93A;

IKK $\beta^{f/wt}$; CSF-1R-cre⁺ mice compared to cre⁻ controls. While disease onset was not altered (101 days in cre⁻ and 100 days in cre⁺), disease progression was delayed by 19.5 days in cre⁺ mice which is 51% longer than controls (Figure 5A, 5B and 5C).

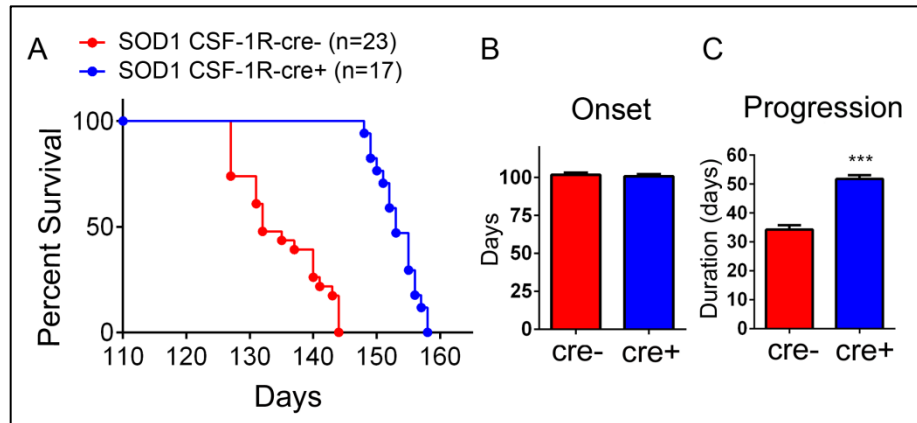


Figure 5. NF-kB inhibition in microglia extends survival in SOD1-G93A mice (A) Kaplan-Meier survival curve of SOD1-G93A; IKK $\beta^{f/wt}$; CSF1R-cre⁺ and SOD1-G93A; IKK $\beta^{f/f}$; CSF1R-cre⁻ mice confirm NF-kB suppression in microglia extends survival by slowing disease progression (C) and not by altering disease onset (B).

Conclusions

In this study we confirm ALS microglia induce motor neuron death in a non-cell autonomous mechanism that we determine is through the classical NF-kB signaling pathway. We show that NF-kB is activated with disease progression in the SOD1-G93A mouse model in astrocytes and microglia. Interestingly, inhibition of NF-kB in astrocytes using transgenic or viral-mediated gene delivery approaches did not improve motor function or survival in SOD1-G93A mice. However, crossing the SOD1-G93A mouse model to a NF-kB-GFP reporter strain revealed NF-kB activation predominately occurs in microglia. Utilizing a robust *in vitro* co-culture model of ALS containing adult primary microglia and embryonic stem cell-derived motor neurons, we show that inhibition of NF-kB in ALS microglia fully rescues motor neurons *in vitro*. Consistent with these results, inhibition of NF-kB specifically in the myeloid lineage *in vivo* significantly delayed disease progression in the SOD1-G93A mouse model by 51%. These data provide a cellular and molecular mechanism by which microglia induce MN death in ALS and suggest a novel therapeutic target to modulate microglial activation and

slow the progression of ALS and other neurodegenerative diseases by which microglial activation plays a role.

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